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(54) Title: COLD-ACTIVE BETA GALACTOSIDASE, THE PROCESS FOR ITS PREPARATION AND THE USE THEREOF

(57) Abstract: A purified cold-active beta galactosidase enzyme specific for lactose, having a stable enzymatic activity at a temperature down to below 8°C.

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Cold-active beta galactosidase, the process for its preparation and the use thereof

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The present invention relates to a purified β-galactosidase specific for lactose.

β-galactosidase catalyzes the hydrolysis of lactose disaccharide into its constituent monosaccharides, glucose and
10 galactose.

This enzyme is widely distributed in numerous .micro-organisms, plant and animal tissues.

The ability of β-galactosidase to hydrolyse lactose into galactose is applied in food industry, particularly in the field of dairy
15 products because of the nutritional (lactose intolerance), technological (crystallisation) and environmental (pollution) problems associated with lactose (Triveni P.S., 1975, CRC Critical Reviews in Food Technology 325-354). The added value gained by the hydrolysis of lactose, to its constituent monosaccharides glucose and galactose, lies in the
20 increased usefulness of hydrolysed lactose as a food carbohydrate. Lactose itself has limited use in this respect because of its relatively low sweetness, solubility and digestibility, but the hydrolysis products of lactose, i.e. glucose and galactose, are superior in all of these respects. Increased sweetness and solubility improve the technical usefulness of

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whey products while the increased digestibility of hydrolysed lactose also offers the opportunity of supplying milk solids to populations which have hitherto been unable to consume milk products because of their inability to hydrolyse lactose in the digestive tract.

5   The β-galactosidase can be applied to the production of low-lactose milk and in the production of galactose or glucose from lactose contained in milk serum which is formed in large amount in the process of producing cheese.

         The major applications for lactose hydrolysis are listed

10 below.

a) Liquid milk. Lactose hydrolysis in liquid milk improves digestibility for lactose intolerant consumers. In flavoured milks, lactose hydrolysis increases sweetness and enhances flavours.

b) Milk powders. Lactose hydrolysed milk powders for dietetic uses,
15 especially for infants with temporary β-galactosidase deficiency.

c) Fermented milk products. In some cases, lactose hydrolysis in milk used for the manufacture of cheese and yoghurt can increase the rate of acid development and thus reduce processing time.

d) Concentrated milk products. Lactose hydrolysis in concentrated milk
20 products (e.g. sweetened condensed milk, ice cream) prevents crystallisation of lactose.

e) Whey for animal feed. Lactose hydrolysis in whey enables more whey solids to be fed to pigs and cattle and also prevents crystallisation in whey concentrate.

25 f) Whey. Lactose hydrolysed whey is concentrated to produce a syrup containing 70-75 per cent solids. This syrup provides a source of functional whey protein and sweet carbohydrate and is used as a food ingredient in ice cream, bakery and confectionery products.

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The conventional approach in food processing is to carry out the hydrolysis of lactose at 40°C during approximately four hours. (T. Godfrey and J. Reichelt in : "Industrial Enzymology : the application of enzymes in industry"; The Nature Press, Mac Millan Publishers Ltd, GB, 1983). However, milk or lactose solution as a raw material is a preferable nutrition source for bacteria. As the result, the putrefaction owing to the saprophyte contamination during the treatment is a serious problem in the food production. Thus, the fact is that the conventional β-galactosidase is not put into practical use.

Attempts to solve these problems consisted in using thermophilic enzymes as described in U.S. Patent 4237230 and U.S. Patent 4007283 but a problem of high energetic cost still remains.

On another hand, cold-adapted β-galactosidases have been studied (Trimbur D.E. and al., 1994; Appl. Environ. Microbiol. 60:4544-4552; Rahim K. A. A. and Leb B. H., 1991, Biotechnol and Appl. Biochem., 13, 246-256).

However, these β-galactosidases generally known in the prior art, when used for food processing, all have one or more disadvantages such as low enzyme activity and low stability at a temperature below 20°C, narrow range of optimum pH and the inhibition of enzymatic action by a reaction product, such galactose or others products particularly calcium.

The object of the present invention is to hydrolyse lactose by using a β-galactosidase, which could overcome the above-mentioned drawbacks which are usually associated to this process, while advantageously avoiding contamination problems during the hydrolysis process and lowering the energy consumption.

This problem is solved according to the present invention by a purified cold-active β-galactosidase, specific for lactose, having a

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stable enzymatic activity at temperatures up to below 8°C, preferably up to below 6°C, and specifically at 4°C, which corresponds to refrigerating conservation temperature for dairy products. This enzyme of the invention is consequently able to hydrolyse lactose in dairy products and
5   milk processing at such a low temperature that saprophytes are hindered to proliferate. The hydrolysis of lactose can be carried out in these refrigeration conditions with no need of a particular treatment to the dairy product concerned.

According to the invention, an enzymatic activity is
10   considered as stable when, in the concerned conditions, the enzyme is capable of lasting long enough to obtain the desired effect, for example, the hydrolysis of a substrate.

According to an embodiment of the invention, the cold-active β-galactosidase has a stable enzymatic activity between 0 and
15   50°C.

Advantageously, the cold-active β-galactosidase according to the invention has a stable enzymatic activity at a pH range from 6 to 10, preferably from 6 to 8.

Preferably, the cold-active β-galactosidase according to the
20   invention has a stable enzymatic activity in presence of calcium and/or galactose, meaning that the activity of this enzyme is neither inhibited by its reaction product nor by products being present in milk. This property allows to use efficiently this enzyme in milk treatment.

Such a cold-adapted β-galactosidase according to the
25   invention attains the level of practical application, having simultaneously the following properties :

(1) Having a sufficient stability in the neighbourhood of 0 to 10°C

(2) Having a sufficient enzymatic activity at a pH range from 6 to 10

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(3) Having an enzymatic activity non inhibited by reaction products or other products substantially present in milk, such calcium.

According to an advantageous embodiment of the invention, the enzyme can be inactivated at a pasteurisation temperature. This property of the enzyme according to the present invention allows to apply the β-galactosidase according to the invention and to stop the enzymatic reaction of lactose hydrolysis without any additional step during a current milk treatment.

Another object of the present invention is a strain of an isolated psychrophilic bacterium capable of producing a cold-active β-galactosidase according to the present invention. A preferable strain is *Pseudoalteromonas haloplanktis* deposited on the 4th of November, 1999, under the Budapest Treaty at the Belgian Coordinated Collections of Microorganisms (BCCM™), Laboratorium voor Microbiologie - Bacteriënverzameling (BCCM™/LMG), Universiteit Gent, K.L. Ledeganckstraat 35, 9000 Gent, Belgium, with the Accession N° LMG P-19143 and variants and mutants derived therefrom.

To purify a cold-active β-galactosidase according to the invention, a bacterium living in the Antarctic area was isolated and characterised in order to study how its enzymes, and particularly, the β-galactosidase was adapted to cold. These studies led to the purification of the β-galactosidase, meaning that this protein was obtained substantially free of other proteins as determined by Sodium Dodecyl Sulphate Polyacrylamide gel Electrophoresis (SDS-PAGE) using protein purification steps known in the art.

Micro-organisms can be divided in categories depending on the temperature at which they can proliferate. The widely accepted definition by Morita (Psychrophilic bacteria. Bacteriol. Rev. 39: 144-167; 1975.) proposes that psychrophiles include organisms having optimum

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growth temperatures <15°C and upper cardinal temperatures around 20°C, although they are able to multiply and to carry out all their biochemical functions near the normal freezing point of water. The mesophilic bacteria proliferate at an average temperature range between 5   25 and around 40°C. Thermophilic micro-organisms proliferate at a temperature above 50°C and hyperthermophilic micro-organisms grow at temperatures above 80°C.

It is still an object of the present invention to provide a DNA sequence comprising a gene which encodes a polypeptide having the biological activity of the cold-active β-galactosidase according to the 15   invention. A preferable DNA sequence is shown in SEQ ID N°1 and a polypeptide having an amino acid sequence as shown in SEQ ID N°2 is preferable.

Another object of the invention is a recombinant plasmid suited for transformation of a host, capable of directing the expression of 20   a DNA sequence according to the invention in such a manner that the host expresses said polypeptide having the biological activity of the cold-active β-galactosidase in recoverable form. According to the invention another object is the so transformed host.

A variety of host-expression systems may be conceived to 25   express the cold-active β-galactosidase coding sequence, for example bacteria, yeast, insect cells, plant cells, mammalian cells, etc.

Particularly, in yeast, a number of vectors containing constitutive or inducible promoters may be used. For a review see Grant and al., 1987, Expression and secretion vectors for yeast, in Methods in

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Enzymology, Eds. Wu & Grossman, 31987, Acad. Press, N.Y., Vol. 153, pp. 516-544.

It is also an object of the present invention to provide a process for purifying the cold-active β-galactosidase according to the
5   invention from a psychrophilic bacterium as well as to provide a process for producing cold-active β-galactosidase according to the invention in a transformed host.

These and other objects of the present invention will be apparent from the following disclosure.

10   Other characteristics of the present invention are listed in the annexed claims.

Fig. 1a shows the growth of the strain *Pseudoalteromonas haloplanktis* LMG P-19143 at different temperatures.

Fig. 1b shows the cell viability and the cold-active
15   β-galactosidase activity of the strain *Pseudoalteromonas haloplanktis* LMG P-19143 versus temperatures.

Fig. 2 shows the effects of divalent metal ions on β-galactosidase activity from *E. coli* and *Pseudoalteromonas haloplanktis* LMG P-19143.

20   Fig. 3 shows effect of β-mercaptoethanol on kcat of β-galactosidase from *E. coli* and *Pseudoalteromonas haloplanktis* LMG P-19143 with ONPG as a substrate.

Fig. 4 shows the specific activities of β-galactosidase from *E. coli* and *Pseudoalteromonas haloplanktis* LMG P-19143, between 8
25   and 60°C, using ONPG as a substrate.

Fig. 5 shows the thermal stability of the activity of β-galacosidase from *E. coli* and from *Pseudoalteromonas haloplanktis* LMG P-19143 at 45°C, using ONPG as a substrate.

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Fig. 6 shows the thermo-dependence of the physiological efficiency (kcat/km) of β-galactosidase of *Pseudoalteromonas haloplanktis* LMG P-19143, using ONPG as a substrate.

5   <u>Screening of a bacterial strain and culture conditions.</u>

A bacterial strain was isolated and selected from sea water on necrosed algae at the J.S. Dumont d'Urville Antarctic Station (60°40'S; 40°01'E) The strain was identified as a *Pseudoalteromonas haloplanktis* by identification systems such the quantitative analysis of

10   cellular fatty acid composition performed using a gas-liquid chromatography procedure known in the Art (Mergaert et al, 1993, Int. J. Syst. Bacteriol., 43, 162-173), using the Microbial Identification System (MIS, Microbial ID Inc., Newark, Delaware, U.S.A.). The peak recognition program was the MIS TSBA40 database for fatty acids and the

15   chromatographic profiles were identified by comparison to the MIS TSBA database for aerobic bacteria (version 4.0).

The identified strain was then deposited according to the Budapest Treaty at the BCCM (Belgian Coordinated Collections of Micro-organisms) with the following accession Number : LMG P-19143, on the

20   4th of november, 1999.

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The screening of strains collected in the Antarctic, for showing a β-galactosidase activity, was carried out on L-agar plates containing 10g/l bactotryptone, 5g/l yeast extract, 25g/l sea salts, 17g/l

5   agar (Difco) with 0,2% lactose, 32mg/l X-Gal (5- Bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Eurogentec) with or without 1mM IPTG (isopropyl-thio-β-D-galactopyranoside) (Sigma); Growth properties were studied in L-Broth (10g tryptone, 5g yeast extract, 30g sea salts in 1L at pH8,5) containing 1% or 2% lactose. Cultures inoculated with 10ml of a

10   pre-culture grown at 4°C were run at 250 rpm in 500 ml Erlenmeyer flasks containing 300 ml culture medium. After 115 hours culture, the absorbance of the culture was measured at 550 nm and the cells were pelleted and sonicated.

The definition of enzyme activity units can be defined

15   according to the substrate used : with lactose as a substrate, the unit of activity is defined as the amount of enzyme which releases one micro-mole glucose in one minute under standard reaction conditions (temperature, pH). Another commonly used substrate is ortho-nitrophenyl-β-D galactopyranoside (ONPG) and in this case, the unit of

20   activity is defined as the amount of enzyme which hydrolyses one micro-mole of ONPG in one minute under standard reaction conditions.

The degree of hydrolysis, defined as the percentage of lactose molecules cleaved, is most simply measured by determination of the amount of glucose released, or by changes in the physical properties

25   of the hydrolysed lactose solution. Solution properties such as freezing point depression change as the disaccharide lactose is converted into the lower molecular weight monosaccharides glucose and galactose.

The intracellular β-galactosidase activity was assayed using ONPG as substrate. When 1mM IPTG was added to the culture,

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the β-galactosidase activity was enhanced at least 2 times in the strain selected among the bacterial samples collected. This selected strain was a Gram negative and protease positive bacterium, chosen for its high β-galactosidase activity and its growth properties in liquid medium.

5   The strain was characterised and different growth conditions were tested. Sea salts at different concentrations were added to the culture medium: 5, 10, and 30 g/l with a lactose concentration of 10 or 20g/l. The optimum growth medium was a rich medium comprising 2% lactose and 3% sea salts. In particular, the addition of sea salts to

10   the growth medium enhanced the growth of the strain by a factor of ten.

The effects of adding IPTG in the growth medium were also studied and three IPTG concentrations were tested: 0.1 mM, 1 mM and 10 mM. It was observed that the addition of 1 mM IPTG to the growth medium after 44 hours of culture doubles the β-galactosidase activity in

15   the cells.

Fig. 1a shows the growth rates of the strain at four temperatures : 4°C, 12°C, 18°C and 25°C by measuring the absorbance of the culture at 550 nm. The results obtained showed that temperatures above 4°C induced faster growth rates but in the same time, reduced

20   strain development. It is worth mentioning that growth rates are inaccurate as a sole criterion to determine the optimal growth temperature. This is clearly illustrated by the Fig. 1b showing the cell viability and the β-galactosidase activity of the strain of the invention at different temperatures.

25   The β-galactosidase from *Escherichia coli* used as a control was from Sigma (G2513).

The assay of β-galactosidase was carried out using 3mM ONPG (ortho-nitrophenyl-β-galactopyranoside) as a chromogenic substrate in 100 mM sodium phosphate buffer, pH 7,3, 1 mM MgCl$_2$, 100

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mM 2-mercaptoethanol (Sigma). Activities toward the chromogenic substrate were recorded in a thermostated Uvicon 860 Spectrophotometer (Kontron) at 25°C and calculated on the basis of an extinction coefficient for o-nitrophenol of 3.5 mM^{-1} cm^{-1} at 410 nm (Miller,

5   J.H. and Reznikoff, W.S., Eds. 1978; The Operon. Cold Spring Harbor Laboratory Press, NY.). Assays using lactose as a substrate were carried out using various concentrations of lactose. The reaction was stopped by boiling the sample in a water bath for 3 minutes. The galactose dehydrogenase assay was used to measure the amount of galactose

10   released by the enzyme (Schachter H. 1975, Enzymatic microassays for D-Mannose, D-Glucose, D-Galactose, L-Fucose, and D-Glucosamine. Methods Enzymol., 41: 3-10.) The specific activity of β-galactosidase is defined as micro-moles of galactose released per minute per mg of protein.

15

Purification and characterization of a cold-active β-galactosidase from the strain LMG P-19143 of the present invention

The Antarctic strain was cultivated at 4°C for 5 days in ten litres of LB broth containing 2% lactose. After 44 hours, the culture was induced

20   by ITPG (isopropyl-L-thio-β-D-galactopyranoside) to a final concentration of 1 mM and left for 68 hours.

The cells were harvested by centrifugation at 12,000 x g for 60 minutes at 4°C and re-suspended in 200 ml 50 mM MOPS (3-morpholinopropanesulfonic acid) buffer, pH 7,5. The cell-free extract

25   was prepared by cell desintegration using the disruptor (LH-SGI Inceltech). 1 mM PMSF (Phenyl-methyl-sulphonyl-fluoride) was added to the crude extract to neutralise serine active proteases and debris were removed by centrifugation at 15,000 x g for 30 minutes. Supernatant was then treated for two hours by protamine sulphate at a final concentration

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of 1 g/l to remove nucleic acids. After centrifugation for 30 minutes at 27000 x g, the supernatant wad dialysed against 2 x 2 litres of MOPS buffer and then loaded on a DEAE-agarose column (35 x 2.5 cm) equilibrated in MOPS buffer and eluted with a NaCl linear gradient

5   (500 ml-500 ml, 1M, NaCl). Fractions containing β-galactosidase activity were pooled, concentrated up to 20 ml and dia-filtrated againts MOPS buffer using a Minitan tangential flow ultra-filtration unit (Millipore) fitted with PTHK membrane (100 kDa molecular mass limit). The sample was then added to an affinity matrix of agarose derivatized with

10   p-aminobenzyl-1-thio-β-D-galactopyranoside   (Sigma   A0414) (Steers E., Jr., Cuatrecasas P., and Pollard H., B., 1971, J. Biol. Chem. 246:196-200). The matrix containing the sample was washed with 1M KCl and eluted with 100 mM lactose in MOPS buffer containing 1M KCl. The active fractions were pooled and applied on a Sephacryl S-300

15   column (95 x 3 cm) eluted with MOPS buffer.

Several steps were necessary to purify to homogeneity β-galactosidase from LMG P-19143. These steps are summarised in Table 1.

20   TABLE I

Purification of the intracellular β-galactosidase from LMG P-19143

One unit of β-galactosidase is defined as the amount of the enzyme required to release 1 μmole of nitrophenol min at pH 7.3 and at 20°C

Purification step	Vol (ml)	Protein (mg)	Total activity (μmol/min)	Sp act (μmol/min/mg)	Recovery (%)	Purification (fold)
Crude extract	200	1480	6954	4.7	100	1
DEAE-agarose	80	178.4	3101	1704	45	3.7
Affinity Chromatography	6	9.1	2509	276.5	36	58.8

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Upon loading on DEAE sepharose column, β-galactosidase was eluted as a single peak at a NaCl concentration of approximately 400 nM. Although the affinity column decreased the yield of active

5   β-galactosidase, it increased the purity by removing other remaining contaminant proteins. From 2 L culture grown under the conditions described above, the yield of purified β-galactosidase amounted to 10 ng. Following this procedure, the enzyme is 99% pure as determined by SDS-PAGE and has an estimated apparent molecular mass of

10   118 kDa. Ultrafiltration tests showed that β-galactosidase from LMG P-19143 is concentrated by an ultra-filtration membrane displaying a cut off of 300 kDa.

Analytical procedures

15   Protein concentrations were determined by the method of Bradford (Bradford, M. M., 1976, Anal. Biochem. 72:248-254) using reagents from Pierce and bovine serum albumin as standard. For the purified enzyme, the following extinction coefficients at 280 nm were used : β-galactosidase from *E. coli*; 241590 M^{-1} cm^{-1}, β-galactosidase

20   from LMG P-19143; 195000 M^{-1} cm^{-1}.

The NH_2-terminal amino acid sequence of the LMG P-19143 β-galactosidase was determined using a pulsed liquid phase protein sequencer (Procise Applied Biosystems 492).

SDS-polyacrylamide gel electrophoresis and isoelectric focusing

25   were run essentially as described by the supplier of the electrophoresis equipment (Hoeffer Scientific Instruments). Isoelectric experiments were carried out using pH ranges 3.5-10 and 4-6 in 6% polyacrylamide gels containing 5.5% ampholytes. The anolyte was 0.02 M acetic acid and the catholyte was 0.02M NaOH.

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The activation energy (E_a) was determined from the slope ($-E_a/R$) of Arrhenius plot and the thermodynamic activation parameters of the reaction were calculated according to the following equations :

$$\Delta G^* - \Delta H^* - T\Delta S^* \qquad\qquad\qquad \text{(Eq. 1)}$$

$$\Delta H^* = E_a - RT \qquad\qquad\qquad \text{(Eq. 2)}$$

$$\Delta S^* = 2.303\ R(\log k_{cat} - 10.753 - \log T + E_a\ 2.303\ RT) \qquad \text{(Eq. 3)}$$

The isoelectric point of the β-galactosidase from LMG P-19143 was determined at 7.8; this value is higher than that of *E. coli* β-galactosidase which was found to be 4.6 [Wallenfels K. and Weil R., 1972, In "The enzymes" (Boyer, P.D., ed) Academic Press, New York 7:617-663].

To determine the optimal pH, the enzyme activity was measured in Michaelis's barbital sodium acetate buffer with pH values from 3 to 9.5 and Sorensen's glycin II buffer with pH values from 8.5 to 13. The pH optimum for the LMG P-19143 β-galactosidase activity was found to be at pH 8.5 which is slightly higher than that of *E. coli* enzyme. Over a pH range from 6.5 to 10, both mesophilic and psychrophilic enzymes retain 90% activity after 90 minutes and 60% after 24 hours exposure. The pH stability was optimum at pH 9. The stability of LMG P-19143 β-galactosidase was also tested in various buffers 50 mM MOPS, MES, TRIS and CHES at different pH values (from 5.5 to 9.7) for 20 hours. The enzyme stability is better in MOPS buffer at pH 7.5 and in MES buffer at pH 7.

The effect of various cations such as Zn^{2+}, Mn^{2+}, Cu^{2+}, Ni^{2+}, Li^{2+}, Co^{2+}, Ca^{2+}, Na^{2+} and Fe^{2+} on the enzyme stability was also investigated. The activity of the enzyme was measured at time zero and then after 1,

15

2, 4 and 29 hours incubation at 4°C. The enzyme is stable in the presence of 0.1 to 1 mM Mg^{2+} and also in 0.1 mM Li^{2+} and 0.1 mM Ca^{2+}. The LMG P-19143 β-galactosidase is inhibited by Cu^{2+}, Ni^{2+} and Zn^{2+} at concentrations from 0.1 to 10 mM and by 10 mM Fe^{2+}.

5   To determine the effect of divalent metal ions on activity, assays were performed in 100 mM phosphate buffer at 25°C and pH 7.5. The enzyme preparation was treated with 5 mM EDTA to complex metal ions. After this treatment, the enzyme showed less than 10% of its initial activity. Addition of 10 mM magnesium restored and enhanced two times 10   the activity of LMG P-19143 β-galactosidase, 20 mM calcium or 5 mM manganese restored partially the activity of the enzyme. Addition of 20 mM Mg^{2+} restored the activity of E. coli β-galactosidase, 20 mM Ca^{2+} or Mn^{2+} 5 mM restored partially the activity of E. coli β-galactosidase as shown in Fig. 2.

15   The effect of K+ was determined by assaying activity in 100 mM phosphate buffer containing KCl at concentrations from 0 to 100 mM. LMG P-19143 β-galactosidase optimal activity was recorded using a KCl concentration of 80 mM whereas the E. coli β-galactosidase optimal activity was recorded at a KCl concentration of 40 mM. At theses 20   concentrations, KCl stimulated the activity of both enzymes by a factor of 1.5.

Fig. 3 shows the effect of 2-mercaptoethanol on β-galactosidase activity evaluated in the same conditions. Optimal activity of the LMG P-19143 enzyme was recorded at 80 mM 2-mercaptoethanol and that of 25   E. coli enzyme at 40 mM 2-mercaptoethanol. At these concentrations, the reducing agent stimulated LMG P-19143 enzyme activity twofold and E. coli enzyme by a factor of 1.5.

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Fig. 4 shows the effect of temperature on the β-galactosidase activity determined by assaying the enzyme at various temperatures from 5°C to 60°C using ONPG as a substrate. The thermo-dependency of the activity of LMG P-19143 β-galactosidase shows a shift of the apparent

5   optimal temperature of activity by 10°C toward low temperatures when compared to the E. coli enzyme. At 8°C, the kcat (s^{-1}) of the LMG P-19143 enzyme is twice as high as that of E. coli enzyme. Theses curves have been used to construct Arrhenius plots and to calculate the activation energy parameters of the reaction as shown in table 2.

· 10

TABLE 2

Kinetic and thermodynamic activation parameters of β-galactosidase
activity at 20°C using ONPG as substrate

Parameter	LMG P-19143	E. coli
k_{cat} (s^{-1})	408	199
E_a (kJ mol^{-1})[a]	15.5	36.2
ΔG^* (kJ mol^{-1})	60.5	62.4
ΔH^* (kJ mol^{-1})	13.1	33.8
ΔS^* (J mol^{-1} K^{-1})	-162	-97.6

15

The lower free energy of activation (ΔG^*) of LMG P-19143 β-galactosidase correlates well with its higher specific activity, but the contribution of the enthalpy term (ΔH^*) and of the entropy ($T\Delta S^*$) to ΔG^* also differs in both enzymes.

20   Thermal stability was determined by incubating the enzymes at different temperatures and periodically withdrawing for assay at 25°C. Fig. 5 shows that, at 45°C, the half-life of the LMG P-19143 β-galactosidase (30 min.) is 12 times lower than the half-life (6 hours) of the E. coli enzyme.

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Assays were performed with ONPG as a substrate at various concentrations and at different temperatures to determine Km and V_{max} values. At 10°C, the apparent Km is nearly the same for the two enzymes. Moreover, as shown in Fig. 6, the physiological efficiency

5   (kcat/km) is about three times higher for the LMG P-19143 β-galactosidase.

Km was also determined at 25°C with lactose (1 mM to 50 mM) as a substrate. Apparent Km was 2,4 mM with the LMG P-19143 enzyme and 13 mM with E. coli enzyme. LMG P-19143 β-galactosidase displays

10   a kcat of 34,1 U/mg and the E. coli enzyme a kcat of only 2,15 U/mg. The physiological efficiency (kcat/km) of the cold-adapted enzyme is ninety times higher than that of the E. coli β-galactosidase.

The β-galactosidase of the present invention being purified and its physiological properties being established, a further step was to

15   investigate the genetic characteristics of it.

DNA isolation

DNA from strain LMG P-19143 was isolated by a modification of the method of Brahamsha, (Brahamsha B., and E. P. Greenberg, 1987,

20   J. Bacteriol. 169:3764-3769). The lysozyme concentration was increased to 1 mg/ml and the cells were treated for 30 minutes at 37°C. The extract was then incubated in 0.5% sodium dodecyl sulphate (SDS) and proteinase K (1 µg/ml final) at 55°C for one hour. The resulting lysate was then extracted three times with an equal volume of

25   phenol/chloroform (50% V/V) followed by a chloroform extraction. The DNA was then precipitated with ethanol and suspended in TE buffer (10 mM Tris. Cl, 1 mM EDTA, pH 8).

Cloning

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The restriction and ligation enzymes were supplied by Gibco and BMI. Genomic DNA of LMG P-19143 extracted according to the protocol described above, was digested with Sau 3AI, Hind III, Pst I or Sph I and the resulting fragments were inserted into the corresponding sites of the

5   plasmid pSP 73 (Promega). The ligated DNA were transformed in *E. coli* dH5α whose endogeneous β-galactosidase is inactived by mutation. Indeed, the plasmid pSP 73 lacks a portion of the lac Z gene which provides essential α-complementation for endogeneous β-galactosidase of *E. coli* dH5α. The plasmid pSP 73 is directly derived from pBR 322

10   (Promega, USA). It displays an oligonucleotides sequence of 2464 pb (GenBank : EMBL accession number X65333). The transformants were selected on L-agar plates containing 50 μg ampicillin/ml, 0.01% X-Gal (5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside) and 100 μM IPTG. After two days incubation at 25°C, the β-galactosidase-positive colonies

15   became blue. The β-galactosidase gene-containing DNA fragment was subcloned into the polylinker of pSP 73 by digestion with Xba I, Bgl II or Eco RI and plasmid self ligation. The sub-clones were analysed by testing β-galactosidase activity on L-agar plates containing 50 μg ampicillin/ml, 0.01% X-gal and 100 μM IPTG.

20   For DNA sequencing, the sub-clone Eco RI was ligated in pK 19 (Pridmore R.D., 1987, Gene 56:309-312). DNA sequencing was performed using the chromosome walking technique with 5' Fluorescein labelled primers. The products of the sequencing reaction were analysed on ALF DNA sequencer (Pharmacia, Sweden). Synthetic

25   oligonucleotides used as primers were from Eurogentec S.A.

The N-terminal amino sequence of the purified enzyme according to the present invention has been determined and alignment of the first nineteen amino acids of LMG P-19143 β-galactosidase with the

19

N-terminal sequence of the *E. coli* enzyme showed ten conserved positions :

```
        TSLQH II NRRDWENP I TVQ      LMG P -19143 β-galactosidase
 5         I I        I I I I I I I        I
        DSLAVVLQRRDWENPGVTQ       E. coli β-galactosidase
```

<u>Cloning of the LMG P-19143 β-galactosidase gene</u>

Four genomic libraries of LMG P-19143 DNA were constructed by restriction digestion of DNA with Sau 3AI, Hind III, Pst I or Sph I and
10   ligation into the corresponding sites of the vector pSP 73.

pSP 73 plasmid lacks the lac Zα fragment which could complement the *E. coli* dH5α deleted β-galactosidase. Transformants of *E. coli* dH5α containing the pSP 73 vector without any insert produced white colonies on X-gal plates. From the colonies screened at 25°C,
15   three β-galactosidase positive colonies were obtained. The DNA inserts of these three β-galactosidase positive transformants were the same and this insert is a fragment Pst I-Pst I of neartly 9 kb.

A restriction map of clone Pst I-Pst I was generated and fragments were subcloned to determine the smallest fragment which could encode
20   the β-galactosidase gene. The colonies obtained were analysed on the basis of β-galactosidase activity on X-gal plates. Three clones which produced β-galactosidase activity were found; theses clones were the result of restriction digestion Xba I, Bgl II and Eco RI. The Eco RI fragment was chosen for sequencing.

25

<u>Nucleotide sequence of the LMG P-19143 β-galactosidase gene</u>

The Eco RI-Pst I fragment of 5088 bp has been totally sequenced. A single large open reading frame was found starting with an ATG at nucleotide 1531 and ending with a TAG at nucleotide 4649; it has been

20

sequenced four times on both strands and its sequence is shown in SEQ ID N°1. The first NH_2 terminal amino acids of the native protein determined by EDMAN degradation could be recognised following the ATG of the open reading frame. Therefore the protein corresponds to

5   1038 amino acids with a calculated M_r of 118068. The predicted amino acid sequence of the sequenced gene was compared with protein sequences databases with "BLAST network service" program. The protein sequence shown in SEQ ID N°2 was aligned with *E. coli* lac Z gene by "TFASTA" program with 51% sequence similarities. The LMG

10   P-19143 gene was so designated lac Z on the basis of its sequence similarities with the lac Z from *E. coli*. The alignment showed that the proposed active-site residues in *E. coli* lac Z; Glu-461, Glu-537, Met 502 and Tyr 503 are conserved in the LMG P-19143 sequence. The alignment with other lac Z β-galactosidase showed significant homology

15   surrounding Glu-461 and Glu-537, forming the consensus sequences : W-S-L-G-N-E and I-L-C-E-Y-A-H-A-M-G-N respectively. The tyrosine residue which is important for the reaction is also conserved in the LMG P-19143 sequence.

The β-galactosidase protein sequence analysis had allowed to

20   identify structural features typical of cold-adapted enzymes. For the LMG P-19143 protein, Arginine content (39) and Arg/Arg + Lys ratio (0.47) are smaller than for *E. coli* β-galactosidase, 66 and 0.77, respectively. The proline residues content is also smaller for the cold-adapted enzyme (46 and 62 respectively) and its glycine content was higher within the

25   15 amino acids around the catalytic residue Glu 461.

Alignment with *E. coli* lac Z gene showed three insertions in the LMP-19143 lac Z gene. These insertions of 4, 5 and 9 residues are located at Glu 78, Gln 634 and Asn 739 respectively.

21

The LMG P-19143 β-galactosidase shares structural properties with the mesophilic *E. coli* β-galactosidase. The apparent sub-unit mass of the LMG P-19143 β-galactosidase is comparable to that of *E. coli* enzyme. The cold-adapted enzyme is a multimer since it is concentrated

5   by an ultra-filtration membrane of 300 kDa cut off. The sub-unit is long of 1038 amino acids with a Mr of 118,068, which is slightly higher than that of *E. coli* lac Z enzyme with 1,023 amino acids.

The β-galactosidase from LMG P-19143 shows an optimal pH value of 8.5 for both stability and activity which is comparable to what is

10   observed for the *E. coli* β-galactosidase. The two enzymes have a good activity within the pH range of 6.6-10, this would allow the efficient treatment of milk, the pH of which is 6.6.

LMG P-19143 and *E. coli* β-galactosidase are activated by 2-mercaptoethanol. SH-groups may be involved in the catalytic process

15   but other data show that certain SH-groups may be important for maintaining the active conformation of the enzyme [Wallenfels K. and Weil R., 1972. In "The enzymes" (Boyer, P.D., ed.) Academic Press, New York 7:617-663].

As many fungal and bacterial β-galactosidase, LMG P-19143 and

20   *E. coli* enzymes require divalent cations for activity. Indeed, addition of EDTA, a chelating agent, to the assay mixture leads to enzyme inactivation. Addition of magnesium, calcium or manganese restored the activity. So LMG P-19143 β-galactosidase is a metallo-enzyme having a strict requirement for divalent metal ions as suggested for *E. coli*

25   β-galactosidase by Wallenfels K. and Weil R. in "The enzymes" (Boyer, P.D., ed., Academic Press, New York 7:617-663, 1972). Moreover the three-dimensional structure of *E. coli* β-galactosidase showed two bound magnesium per monomer (Jacobson R.H.,

22

Zhang X-J.,   DuBose R.F.   and   Matthews B.W.,   1994.   Nature 369:761-766).

The alignment of LMG P-19143 sequence with other lac Z β-galactosidase showed the conservation of the amino acid residues
5   involved in catalysis. The proposed mechanism of action for the *E. coli* lac Z β-galactosidase involves a double displacement reaction in which the enzyme forms and hydrolyses a glycosyl-enzyme intermediate via oxocarbonium ion-like transition states (Gebler, J.C., R. Aebersold, and S.G. Withers, 1992. J. Biol. Chem. 267:11126-11130). These authors
10   identified Glu-537 as the nucleophilic amino acid and suggested that Glu-461 serves as the general acid/base catalyst which protonates the galactosyl transition state intermediate and deprotonates the attaching water in the *E. coli* lac Z protein. The analysis of the three-dimensional structure of β-galactosidase from *E. coli* showed that residues Glu 461,
15   Met 502, Tyr 503 and Glu 537 are found closed together and formed a pocket that was identified as the substrate binding site. Glu 537 is situated on the opposite site of the cavity and oriented through hydrogen bonding with Tyr 503 and Arg 388 (Jacobson R.H., Zhang X-J., DuBose R.F. and Matthews B.W., 1994. Nature 369:761-766). These
20   residues are also conserved in the LMG P-19143 sequence. Affinity labelling of β-galactosidase has identified Met-502 as a non-essential active site residue, whereas the suggestion that the adjacent residue, Tyr-503, may play a direct role as an acid/base catalyst, was supported by subsequent analysis of mutants modified at this position (Gebler, J.C.,
25   R. Aebersold, and S.G. Withers, 1992. J. Biol. Chem. 267:11126-11130). Among homologous β-galactosidase sequences, residues that form the active-site pocket are highly conserved (Jacobson R.H., Zhang X-J., DuBose R.F. and Matthews B.W., 1994. Nature 369:761-766).

23

LMG P-19143 β-galactosidase also shares common properties with cold-adapted enzymes (Feller G., Arpigny J.L., Narinx E., and Gerday C., 1997. Comp. Biochem. Physiol. 118A:495-499).

Indeed the cold β-galactosidase displays a lower apparent
5   optimum temperature of activity and a lower thermal stability than the E. coli enzyme. Moreover over the temperature range of 0-40°C, the lever of turnover (kcat) of LMG P-19143 β-galactosidase towards ONPG is higher than that E. coli enzyme. This difference in favour of the cold-adapted enzyme is dramatically increased when lactose is used as
10 substrate (fifteen times at 25°C). The thermodynamic parameters showed in (Table 2) are consistent with the fact that the activated state of the complex is reached through a minimum of entropy change and with a lower activation enthalpy when compared to E. coli β-galactosidase.

With ONPG as a substrate, the km values are, at low temperature,
15 comparable for both enzymes. However, since kcat value is significantly higher to LMG P-19143 β-galactosidase, the physiological efficiency is also higher for the LMG P-19143 β-galactosidase.

With lactose as a substrate at 25°C, km is five times lower for the LMG P-19143 enzyme and the physiological efficiency (kcat/Km) is
20 therefore eighty times as high as that of E. coli β-galactosidase.

The above mentioned data allow to clarify to some extent some questions raised about the possible differences in the molecular adaptation of intracellular enzymes when compared to extracellular ones (Gerday et al., 1998). Indeed in a few cases : citrate synthase and
25 β-galactosidase, the specific activity was not higher than the mesophilic counterparts whereas thermostability was, in all cases, much lower than that of mesophilic enzymes.

The alignment of the amino acid sequence of LMG P-19143 β-galactosidase with that of E. coli β-galactosidase shows three

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insertions of 4, 5 and 9 residues. If located in surface loops, these insertions could contribute to increase the plasticity of the molecular edifice as also suggested in the case of subtilisin S41 (Davail S., Feller G., Narinx E. and Gerday C., 1994, J. Biol. Chem.

5   269:17448-17453). Nevertheless the involvement of indels in cold-adaptation is strongly specific to each enzyme type and can not be generalised (Feller G., Arpigny J.L., Narinx E., and Gerday C., 1997. Comp. Biochem. Physiol. 118A:495-499).

As in the case of several cold-adapted enzymes, the LMG

10 P-19143 β-galactosidase arginine content (55) is lower than that of its mesophilic counterpart (66). Arginine residues play a significant role in thermal adaptation. Indeed, the charge resonance of the guanidium group gives arginine the possibility to form more than one salt bridge (Mrabet et al., 1992) as well as multiple hydrogen bonds with

15 surrounding acceptors (Borders, CL.L, Broadwater, J.A.; Bekeny, P.A., Salmon, J.A., Lee, A.S., Eldrige, A.M., Pett, V.B., 1994, Protein Sci. 3:541-548). The multivalent character of arginine certainly account for its low occurrence in many cold-adapted enzymes and in enzymes of low stability in general (Menendez-Ariaz, M. and Argos, P, 1989, J. Mol. Biol.

20 206:397-406).

The cold-adapted enzyme also shows a lower content of proline (46 compared to 62 in the mesophilic enzyme). The cyclic structure of proline severely impairs the rotations about its N-C^{α} bond. So, the presence of this residue in a protein greatly reduces the number of

25 possible local conformations of the molecular backbone. This reduces the conformational entropy of the unfolded state and confers more rigidity to the native protein (Matthews et al., 1987).

On the contrary, LMG P-19143 β-galactosidase glycine content is lower than that E. coli enzyme. Glycine, which has a side chain,

25

increases the degrees of freedom of the unfolded polypeptide backbone. The replacement of one Gly by another residue in theory can reduce the backbone flexibility and destabilises the unfolded state by as much as 3,3 kJ mol^{-1} at 65°C (Nemethy et al., 1966). Nevertheless it has been

5   suggested that the stacking of Gly around the catalytic residues provides high active site flexibility (Karplus and Shultz, 1985).

To conclude, LMG P-19143 β-galactosidase is a cold-adapted enzyme that is much more active at low and moderate temperatures when compared to the mesophilic enzyme from *E. coli*. Moreover the

10   ideal optimum pH range (6-8) is suitable for lactose hydrolysis in milk and dairy products.

CLAIMS

1.   A purified cold-active beta galactosidase enzyme, specific for lactose, having a stable enzymatic activity at a temperature up to below 8°C.

2.   Purified cold-active beta galactosidase according to claim 1, having a stable enzymatic activity between 0°C and 50°C.

3.   Purified cold-active beta galactosidase according to claim 1 or 2, having an enzymatic activity stable at a pH ranging from 6 to 10.

4.   Purified cold-active beta galactosidase according to anyone of claims 1 to 3, having a stable enzymatic activity in presence of calcium and/or galactose.

5.   Purified cold-active beta-galactosidase according to anyone of the preceding claims, being inactivated at a pasteurization temperature.

6.   Purified cold-active beta galactosidase according to any of the preceding claims, characterized in that it is produced by a strain of a psychrophilic bacterium.

7.   Purified cold-active beta galactosidase according to claim 6, produced by a strain of the psychrophilic bacterium *Pseudoalteromonas haloplanktis* given BCCM Accession N° LMG P-19143.

8.   A psychrophilic bacterium *Pseudoalteromonas haloplanktis* BCCM Accession N° LMG P-19143 and variants and mutants derived therefrom capable of producing a cold-active beta galactosidase defined by any of the claims 1 to 6.

9.   A DNA sequence comprising a gene which encodes a polypeptide having a biological activity of an enzyme beta galactosidase according to any one of claims 1 to 7.

10.   DNA sequence according to claim 9, which is shown in SEQ ID N°1.

11.   DNA sequence which hybridizes with a DNA sequence according to either claim 9 or 10 or is related to the latter by mutation.

12.   DNA sequence according to anyone of claims 9 to 11, which encodes a polypeptide having an amino acid sequence shown in SEQ ID N°2.

13.   DNA sequence according to anyone of claims 9 to 12, which is derived from a genome of a beta galactosidase-forming micro-organism.

14.   DNA sequence according to claim 13, derived from the genome of the psychrophilic bacterium *Pseudoalteromonas haloplanktis* given BCCM Accession N° LMG P-19143.

15.   A recombinant plasmid suited for transformation of a host, capable of directing the expression of the DNA sequence according to claims 9 to 14, in such a manner that the host expresses said polypeptide having the biological activity of said cold-active beta galactosidase in recoverable form.

16.   A host transformed with at least one recombinant plasmid according to claim 15, an expression control sequence in said recombinant plasmid being operatively linked to a DNA sequence in said host.

17.   The host according to claim 16, selected among the group of bacteria and yeast.

18.   A method for purifying cold-active beta galactosidase according to anyone of the claims 1 to 7, which comprises :

inoculating a culture medium having lactose as at least major source of carbon with cells capable of producing said beta galactosidase in said culture medium, said culture medium being suitable for

proliferation of such cells, maintaining conditions of temperature and pH favorable to cell growth and proliferation, harvesting the resulting cells, and extracting the produced beta galactosidase therefrom.

19.   A method for producing a cold-active beta galactosidase
5   according to claims 1 to 7, comprising the steps of :

- extracting genomic DNA from *Pseudomonas haloplanktis* BCCM Accession N° LMG P-19143,

- constructing a recombinant expression vector comprising a gene extracted from said genomic DNA, said gene encoding a
10   polypeptide having a biological activity of said enzyme cold-active beta galactosidase,

- transforming an appropriate host with at least one said recombinant expression vector capable of directing the expression of said polypeptide,

15   - culturing said transformed host, and

- recovering the produced polypeptide having the biological activity of said cold-active beta galactosidase enzyme.

20.   A method for reducing lactose content in dairy products, comprising the step of contacting the dairy product with a purified
20   cold-active beta galactosidase according to anyone of claims 1 to 7, at a refrigeration temperature and at a pH ranging from 6-10.

21.   The method according to claim 20, comprising a supplementary step of heating said contacted dairy product at a pasteurization temperature which inactivates said cold-active beta
25   galactosidase enzyme.
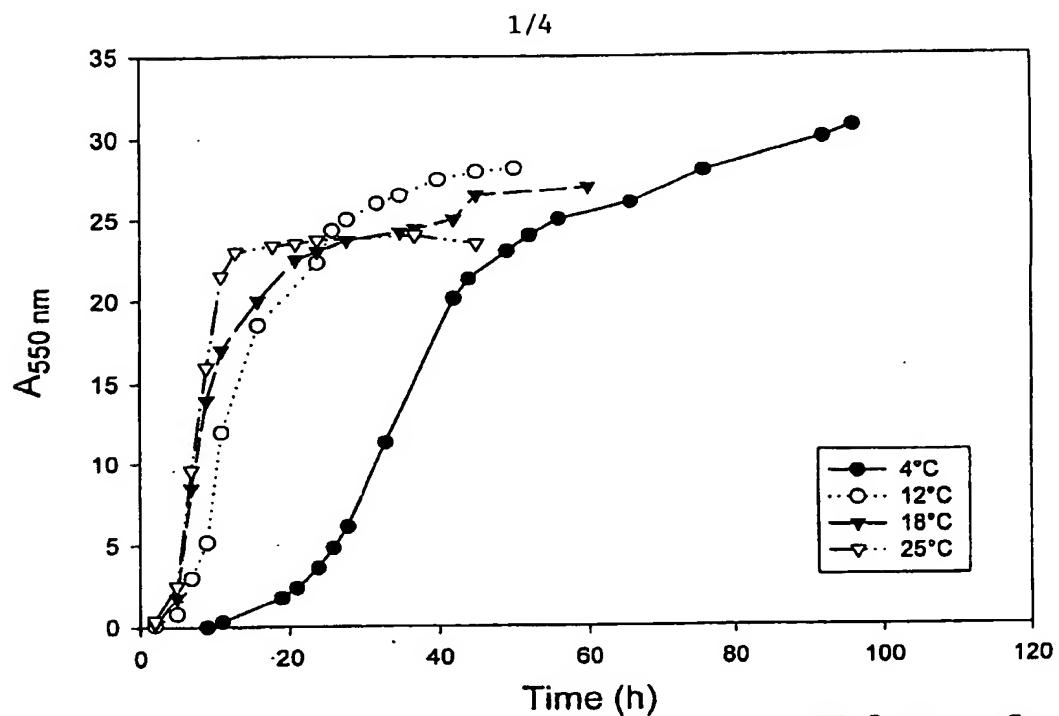
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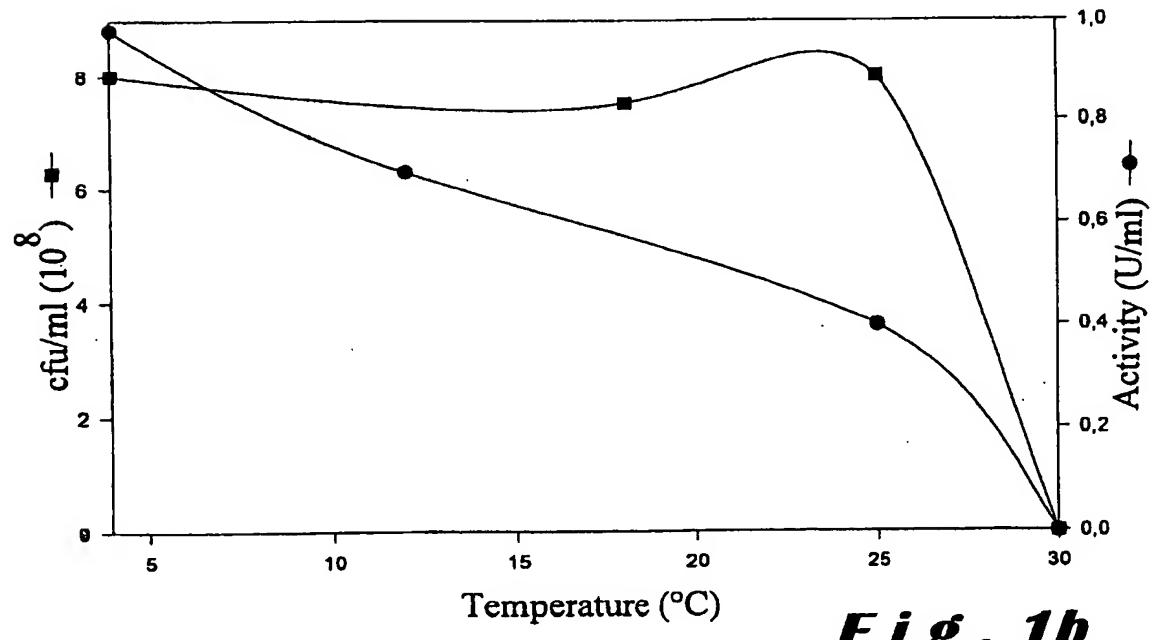
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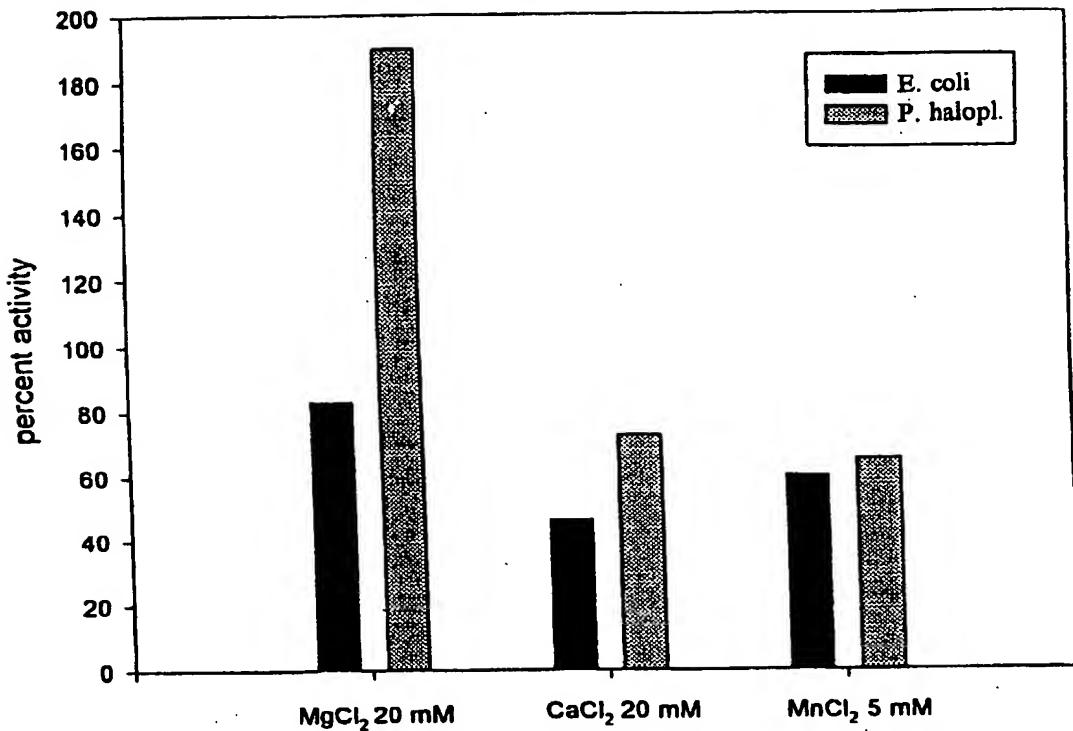
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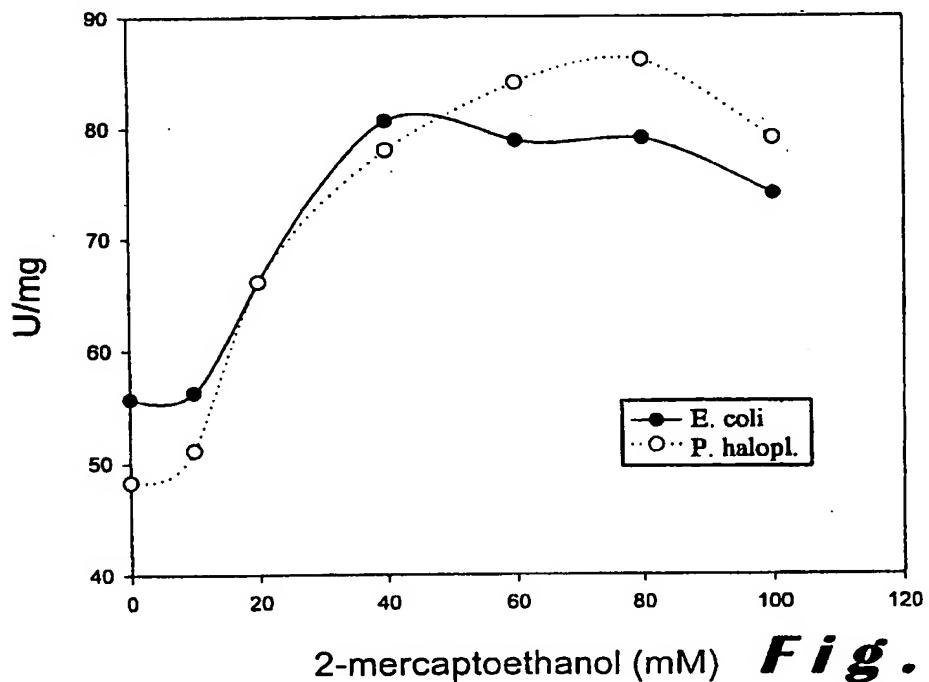
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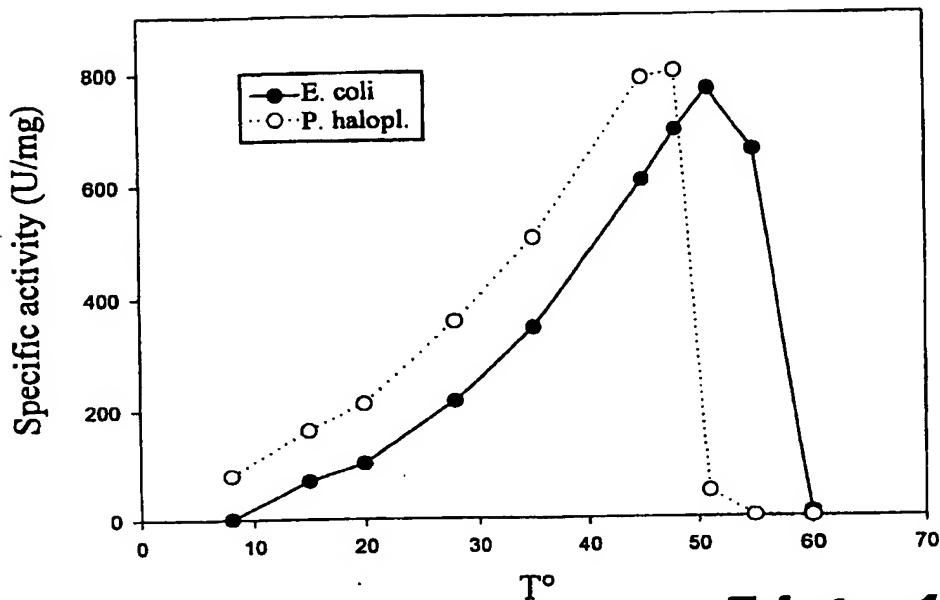
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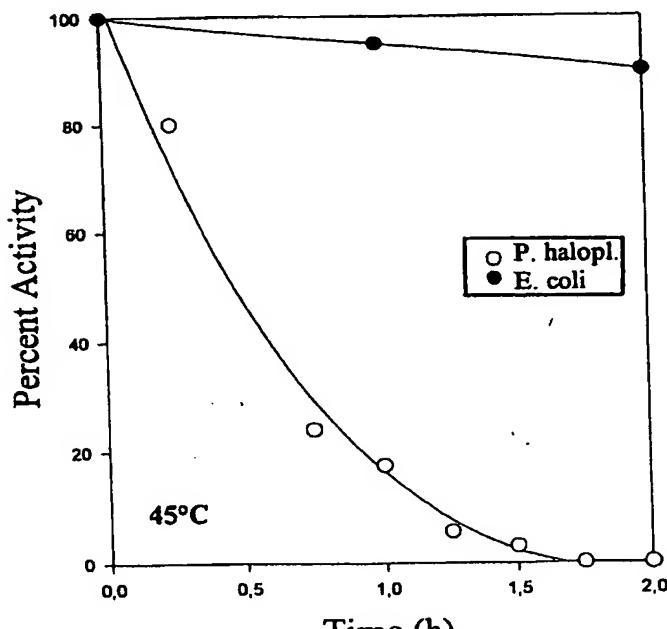
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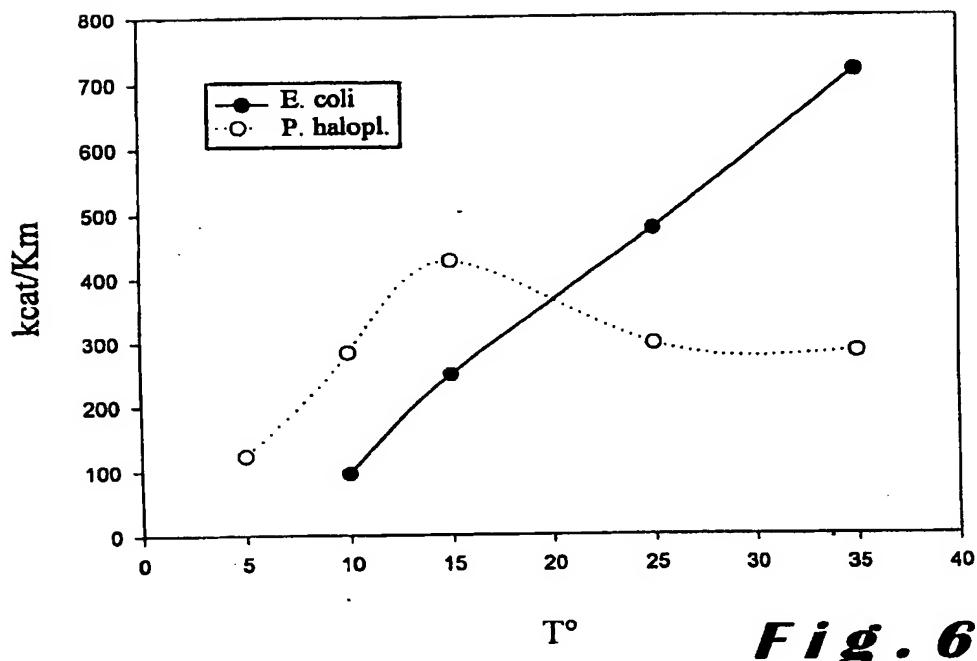
Fig. 6

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Asn Ala Val Arg Thr Ala His Tyr Pro Asn His Pro Leu Phe Tyr Glu
385   390   395   400

Leu Cys Asp Glu Leu Gly Leu Tyr Val Val Asp Glu Ala Asn Ile Glu
                405   410   415

Thr His Gly Met Phe Pro Met Gly Arg Leu Ala Ser Asp Pro Leu Trp
                420   425   430

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Ala Gly Ala Phe Met Ser Arg Tyr Thr Gln Met Val Glu Arg Asp Lys
        435             440             445

Asn His Ala Ser Ile Ile Ile Trp Ser Leu Gly Asn Glu Cys Gly His
    450             455             460

Gly Ala Asn His Asp Ala Met Tyr Gly Trp Ser Lys Ser Phe Asp Pro
465             470             475             480

Ser Arg Pro Val Gln Tyr Glu Gly Gly Ala Asn Thr Thr Ala Thr
            485             490             495

Asp Ile Ile Cys Pro Met Tyr Ser Arg Val Asp Thr Asp Ile Lys Asp
            500             505             510

Asp Ala Val Pro Lys Tyr Ser Ile Lys Lys Trp Leu Ser Leu Pro Gly
        515             520             525

Glu Thr Arg Pro Leu Ile Leu Cys Glu Tyr Ala His Ala Met Gly Asn
    530             535             540

Ser Leu Gly Ser Phe Asp Asp Tyr Trp Gln Ala Phe Arg Glu Tyr Pro
545             550             555             560

Arg Leu Gln Gly Gly Phe Ile Trp Asp Trp Val Asp Gln Gly Leu Ser
            565             570             575

Lys Ile Asp Glu Asn Gly Lys His Tyr Trp Ala Tyr Gly Gly Asp Phe
            580             585             590

Gly Asp Glu Leu Asn Asp Arg Gln Phe Cys Ile Asn Gly Leu Leu Phe
        595             600             605

Pro Asp Arg Thr Pro His Pro Ser Leu Phe Glu Ala Lys Tyr Ser Gln
    610             615             620

Gln His Leu Gln Phe Thr Leu Arg Glu Gln Asn Gln Asn Gln Asn Gln
625             630             635             640

Asn Gln Tyr Ser Ile Asp Val Phe Ser Asp Tyr Val Phe Arg His Thr
            645             650             655

Asp Asn Glu Lys Leu Val Trp Gln Leu Ile Gln Asn Gly Val Cys Val
            660             665             670
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6

Glu Gln Gly Glu Met Ala Leu Asn Ile Ala Pro Gln Ser Thr His Thr
        675   680   685

Leu Thr Ile Lys Thr Lys Thr Ala Phe Glu His Gly Ala Gln Tyr Tyr
    690   695   700

Leu Asn Leu Asp Val Ala Leu Ile Asn Asp Ser His Phe Ala Asn Ala
705   710   715   720

Asn His Val Met Asp Ser Glu Gln Phe Lys Leu Ile Asn Ser Asn Asn
                725   730   735

Leu Asn Ser Lys Ser Phe Ala Ser Ala Thr Glu Lys Ser Val Ile Ser
            740   745   750

Val Asn Glu Thr Asp Ser His Leu Ser Ile Glu Asn Asn Thr Phe Lys
        755   760   765

Leu Val Phe Asn Gln Gln Ser Gly Leu Ile Glu Gln Trp Leu Gln Asp
    770   775   780

Asp Thr Gln Val Ile Ser Ser Pro Leu Val Asp Asn Phe Tyr Arg Ala
785   790   795   800

Pro Leu Asp Asn Asp Ile Gly Val Ser Glu Val Asp Asn Leu Asp Pro
            805   810   815

Asn Ala Trp Glu Ala Arg Trp Ser Arg Ala Gly Ile Gly Gln Trp Gln
            820   825   830

Arg Thr Cys Ser Ser Ile Asn Ala Val Gln Ser Ser Val Asp Val Arg
        835   840   845

Ile Thr Cys Val Phe Asn Tyr Glu Phe Asn Gly Val Leu Gln Ala Gln
    850   855   860

Thr Gln Trp Leu Tyr Thr Leu Asn Asn Thr Gly Thr Ile Ser Leu Asn
865   870   875   880

Val Asp Val Asn Leu Asn Asp Thr Leu Pro Pro Met Pro Arg Ile Gly
            885   890   895

Leu Ser Thr Thr Ile Asn Lys Gln Ser Asp Thr Lys Val Asn Trp Leu
        900   905   910

Gly Leu Gly Pro Phe Glu Asn Tyr Pro Asp Arg Lys Ser Ala Ala Arg
        915   920   925

Phe Gly Tyr Tyr Ser Leu Ser Leu Asn Glu Leu Tyr Thr Pro Tyr Ile
        930   935   940

Phe Pro Thr Asp Asn Gly Leu Arg Ser Asp Cys Gln Leu Leu Ser Ile
945   950   955   960

Asn Asn Leu Ile Val Thr Gly Ala Phe Leu Phe Ala Ala Ser Glu Tyr
                965   970   975

Ser Gln Asn Met Leu Thr Gln Ala Lys His Thr Asn Glu Leu Ile Ala
            980   985   990

Asp Asp Cys Ile His Val His Ile Asp His Gln His Met Gly Val Gly
        995   1000   1005

Gly Asp Asp Ser Trp Ser Pro Ser Thr His Lys Glu Tyr Leu Leu Glu
    1010   1015   1020

Gln Lys Asn Tyr Asn Tyr Ser Leu Thr Leu Thr Gly Gly Ile Thr Thr
1025   1030   1035   1040

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7   C12N9/38

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7   C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL 'Online! ID: UEU131635, 29 December 1998 (1998-12-29) FRANCOIS ET AL.: "A cold adapted bacterial B-galactosidase from Antarctica" XP002137331	1-19
Y	abstract	20,21
Y	WO 92 02143 A (NUTRASWEET CO) 20 February 1992 (1992-02-20) abstract	20,21

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 May 2000	25/05/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Sprinks, M

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9202143	A	20-02-1992	BR	9007703 A	22-06-1993
			EP	0494874 A	22-07-1992
			JP	5501197 T	11-03-1993